BBM-928,* A NEW ANTITUMOR ANTIBIOTIC COMPLEX

III. STRUCTURE DETERMINATION OF BBM-928 A, B AND C

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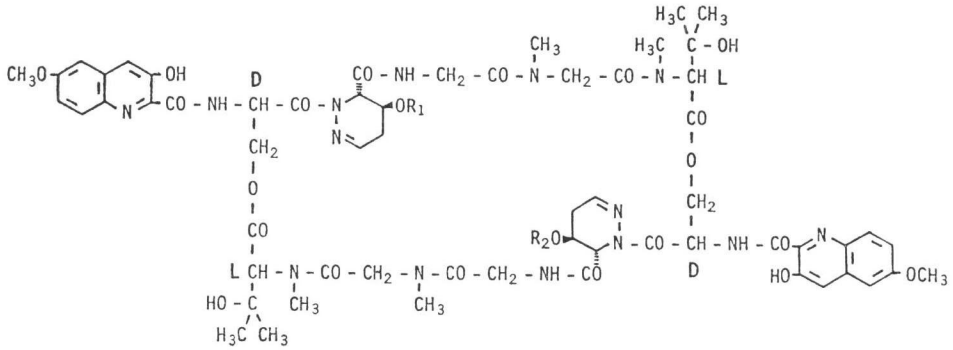
(Received for publication October 30, 1980)

Structures of antitumor antibiotics BBM-928 A, B and C have been determined. They are cyclic decadepsipeptides containing 3-hydroxy-6-methoxyquinaldic acid as a chromophore. Two amino acids, not found in nature, L-β-hydroxy-N-methylvaline and *trans*-(3*S*,4*S*)-4-hydroxy-2,3,4,5-tetrahydropyridazine-3-carboxylic acid, were identified as structural constituents of the antibiotic. In gross structure, BBM-928 resembles the echinomycin group of antibiotics which are cyclic octadepsipeptides having a quinoxaline chromophore, but BBM-928 differs from the latter group by virtue of the lack of a sulfur-containing cross linkage.

BBM-928 is a complex of potent antitumor antibiotics elaborated by a strain of *Actinomadura luzonensis*[1]. The production, isolation, characterization and antitumor activity of BBM-928 have been described in a preceding paper[2]. This paper presents evidence to show that BBM-928 A, B and C possess the following structures:



	R_1	R_2
BBM-928 A	$-COCH_3$	$-COCH_3$
B	$-COCH_3$	H
C	H	H

General Structural Feature and Preliminary Degradation Studies

BBM-928 A, B and C were isolated as colorless crystals and showed physicochemical and spectroscopic properties similar to each other. Nearly identical UV spectra of BBM-928 A, B and C (λ_{max}^{EtOH} at 235, 264 and 345 nm) suggested the same chromophore structure was present in the three components. Acetylation experiments[2] indicated that BBM-928 A and B were, respectively, diacetyl and monoacetyl derivatives of BBM-928 C. Molecular formulae of $C_{64}H_{78}N_{14}O_{24}$, $C_{62}H_{76}N_{14}O_{23}$ and $C_{60}H_{74}N_{14}O_{22}$ were assigned to BBM-928 A, B and C, respectively, based on the results of microanalysis and osmometric molecular weight determination. The molecular weight of BBM-928 A was unequivocally established

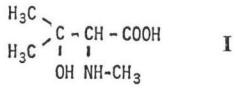
* This antibiotic has recently been named as luzopeptin.

to be 1,426 by field desorption mass spectrometry. The ^{13}C-NMR (CMR) spectrum of BBM-928 A indicated a total of 32 well-defined carbon signals suggesting that the antibiotic consisted of two equivalent halves constituting a sterically symmetric structure. The proton NMR (PMR) supported this assumption, showing the presence of a multiple of nearly 40 protons in the spectrum of BBM-928 A. The PMR and CMR spectra of BBM-928 C also displayed a symmetric structure, whereas those of BBM-928 B indicated an asymmetric conformation.

BBM-928 C was hydrolyzed with 6 N HCl at 110°C for 18 hours in a sealed tube. Lipophilic UV-absorbing material was extracted with *n*-butanol from the hydrolyzate. Amino acid fragments remaining in the aqueous solution were chromatographed on a column of Dowex 50W×4 which was developed by pyridine-formic acid buffer. Four amino acids (**I**, **II**, **III** and **IV**) eluted in that order were isolated and crystallized from aqueous ethanol. Compound **I** is a new amino acid whose structure will be discussed later, while **II**, **III** and **IV** were identified as D-serine, sarcosine and glycine, respectively. The solvent-extractable, UV-absorbing fraction was chromatographed on a silica gel column, yielding two chromophoric compounds, **V** and **VI**.

Structure of I

Molecular formula of $C_6H_{13}NO_3$ was assigned to **I** by microanalysis and mass spectrometry ($M^+ + 1$: m/z 148). The PMR and CMR of **I** indicated two C-CH_3, one N-CH_3, one OH, one COOH, one –CH< and one >C<. The pKa′ values (2.3 and 9.5) suggested an α-amino acid structure for **I**. The presence of a tertiary hydroxyl group was indicated by IR (ν_{OH} 1,160 cm^{-1}) and its reluctance to form acetate. From the above analytical and spectral data, **I** was determined to be β-hydroxy-N-methylvaline. L-Configuration was assigned to **I** based on the clear positive COTTON effects observed in its ORD ($[\phi]_{226.5}$ + 2,420 (peak))[3] and CD curves ($[\theta]_{208}$ +3,503 (peak))[4]. Racemic β-hydroxy-N-methylvaline has been synthesized by IZUMIYA and NAGAMATSU[5], but the optically active isomer has not been described.



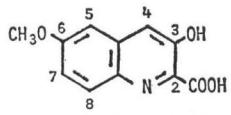
I

Amino acid analysis of the acid hydrolyzate of BBM-928 components indicated that the four amino acids described above (**I**, **II**, **III** and **IV**) were present in equimolar ratio.

Chromophore Structure of BBM-928

Two UV-absorbing fragments, **V** and **VI**, extracted from the acid hydrolyzate of BBM-928 C were acidic compounds and isolated as yellow needles. **V** was analyzed as $C_{11}H_9NO_4$ and its mass spectrum (MS) showed ion peaks at 219 (M^+), 201 ($M^+ - H_2O$) and 175 ($M^+ - CO_2$, base peak). The presence of a phenolic hydroxyl group in **V** was shown by positive $FeCl_3$ reaction. The PMR spectrum of **V** indicated one O-methyl group and four ring protons, and the CMR spectrum included one methyl, one carbonyl and nine aromatic carbons (Table 1). The above information deduced a quinoline or isoquinoline structure for **V** substituted with one member each of –OH, –OCH_3 and –COOH groups. The chemical shifts of the ring protons (δ 6.89–7.66 ppm) indicated that they were not located vicinal to nitrogen, and hence the 2-position of quinoline or the 1- and 3-positions of isoquinoline should be substituted. Upon irradiation of the methyl protons at δ 3.69 ppm, nuclear OVERHAUSER effect (NOE) was observed

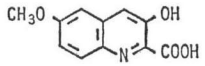
Table 1. PMR and CMR of V.



PMR (60 MHz in D_2O-NaOD)				CMR (in D_2O-NaOD)					
δ (ppm)	M*	J (Hz)	Assignment	δ (ppm)	M*	Assignment	δ (ppm)	M*	Assignment
3.69	s		OCH_3	56.3	q	CH_3	134.5[a]	s	C_{8a}
6.89	d-d	10.5, 1.9	H_7	104.7	d	C_5	155.4	s	C_2
6.91	d	1.9	H_5	116.2	d	C_4	157.5[b]	s	C_3
7.05	s		H_4	118.7	d	C_7	158.4[b]	s	C_6
7.60	d	10.5	H_8	129.0	d	C_8	177.6	s	COOH
				133.2[a]	s	C_{4a}			

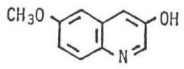
* M: multiplicity; q: quartet; d: doublet; s: singlet.
[a,b] assignment interchangeable.

on two aromatic protons at δ 6.89 and 6.91 ppm (*ca.* 20% enhancement). This observation combined with the multiplicity of ring proton signals indicated that the methoxyl group should be located at the 6-position, and that the 5, 7 and 8 positions were unsubstituted. The CMR spectrum of **V** showed the presence of only one carbonyl carbon (δ 177.6 ppm, assigned to COOH), indicating that the hydroxyl group of **V** should be located in the position not allowing tautomerism to a keto form. Thus, a possibility of an isoquinoline structure for **V** was ruled out, suggesting that the carboxyl and hydroxyl groups should be located at the 2 and 3 positions of the quinoline nucleus, respectively, as shown below:



V

To verify the assigned structure of **V**, **V** was decarboxylated to afford **VII** whose PMR indicated a newly generated H_2 proton at δ 8.36 ppm which coupled with the H_4 proton in the meta position (J= 2.5 Hz).



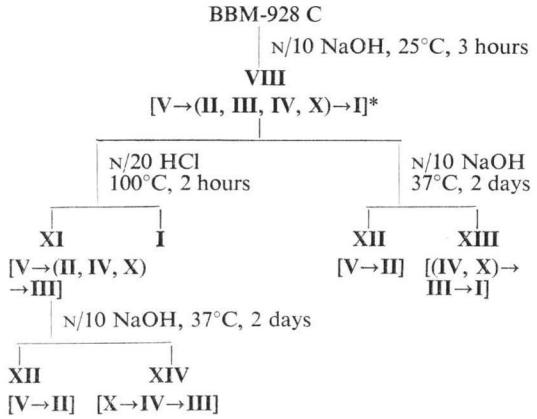
VII

Another chromophoric compound, **VI** ($C_{10}H_7NO_4$), showed properties similar to those of **V** except for the absence of methoxy protons in PMR. **VI** was determined to be 3,6-dihydroxyquinaldic acid, a demethyl derivative of **V**. **VI** is considered to be a secondary degradation product formed during drastic acid hydrolysis.

Controlled Hydrolysis Studies (Chart 1)

Upon treatment with 0.1 N NaOH at 25°C for 5 minutes, BBM-928 A and B were quantitatively deacetylated to afford BBM-928 C which was further hydrolyzed under continued stirring for 3 hours to give a new acidic peptide fragment **VIII**. This compound showed UV and IR spectra similar to those of BBM-928 C except for the absence of ester carbonyl absorption and the appearance of a carboxylate band in the IR spectrum. The presence of free carboxylic acid group in **VIII** was also indicated by the

Chart 1. Controlled hydrolysis of BBM-928 C.



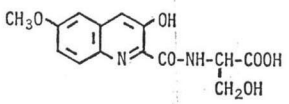
Compound	Structure
I	L-β-Hydroxy-N-methylvaline
II	D-Serine
III	Sarcosine
IV	Glycine
V	3-Hydroxy-6-methoxyquinaldic acid
X	Unknown C_5-unit

* amino acid sequence shown in square bracket

pKa′ determination. Acid hydrolysis of **VIII** in 6 N HCl afforded the same degradation products (**I, II, III, IV, V**) as those obtained from BBM-928 C. **I** was identified to be the C-terminal amino acid of **VIII** by DAKIN-WEST method. Treatment of **VIII** with excess diazomethane afforded a monomethyl derivative **IX** which was analyzed as $C_{31}H_{41}N_7O_{12}$, the formula being supported by its CMR and MS data.

The above results indicated that BBM-928 C was composed of two moles of **VIII**, which were connected with each other by two ester linkages to form a symmetric cyclic structure. From the analytical and CMR data of BBM-928 C and its degradation products, **VIII** (and accordingly BBM-928 C) should have contained an unidentified moiety of C_5-unit in addition to the five structural constituents (**I**～**V**) described earlier. A molecular formula of $C_5H_8N_2O_3$ was assigned for the C_5-unit which will be designated as compound **X** in the following description.

Since **X** could not be isolated as a single entity because of its presumable instability under the hydrolysis conditions examined above, attempts were made to obtain a small peptide fragment which contained **X** in its intact form. Mild acid hydrolysis of **VIII** (N/20 HCl, 100°C, 2 hours) liberated **I** and afforded a new chromophoric fragment **XI** having **III** in the C-terminal. On the other hand, treatment of **VIII** with 0.1 N NaOH at 37°C for 2 days yielded a UV-absorbing fragment **XII** ($C_{14}H_{14}N_2O_6$) and a non-chromophoric tetrapeptide **XIII**. **XII** consisted of **II** and **V** and its structure was determined to be 3-hydroxy-6-methoxyquinaldyl-D-serine.



XII

A similar alkaline hydrolysis of **XI** also liberated **XII** and afforded a tripeptide fragment **XIV**. The above-described reaction scheme is summarized in Chart 1. Comparative analysis of the PMR and CMR spectra of BBM-928 C, **VIII**, **XIII** and **XIV** indicated that the original structure of **X** remained intact in the small peptide fragments, **XIII** and **XIV**, after the above hydrolytic treatment.

Structure Determination of X (Chart 2)

The C-terminal of tripeptide **XIV** should be **III** as in the parent peptide **XI**. **XIV** did not give an N-dinitrophenyl (DNP) derivative indicating that glycine (**IV**) was not the N-terminal. Therefore the amino

acid sequence in **XIV** should be **X**→**IV**→**III**. An authentic sample of glycylsarcosine was prepared by a conventional procedure for comparative purposes. Comparison of the CMR of **XIV** and glycylsarcosine revealed that the following five carbon signals (δ in ppm) were attributable to **X**: >C=O (173.4), –CH= (140.7), 2×–CH< (61.5 and 61.2) and $-CH_2-$ (30.2). As shown in the PMR of **XIV** (Fig. 1), irradiation of methylene protons at δ 2.30 ppm converted a triplet at δ 6.72 ppm into a singlet with concomitant simplification of one of the two methine protons, indicating a partial structure of >CH–CH_2–CH= in **X**. All this structural information led to an assignment of 4-hydroxy-2,3,4,5-tetrahydropyridazine-3-carboxylic acid for the structure of **X**. In order to verify the proposed structure, **XIV** was subjected to bromine oxidation to afford an aromatic compound **XV** ($C_{10}H_{12}N_4O_4$) having UV absorp-

Fig. 1. PMR Spectrum of **XIV** (60 MHz in D_2O).

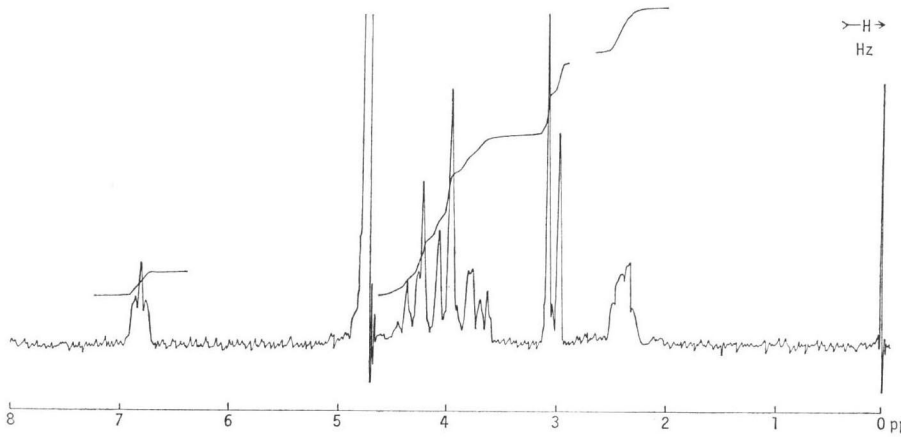


Fig. 2. PMR Spectrum of **XV** (60 MHz in D_2O).

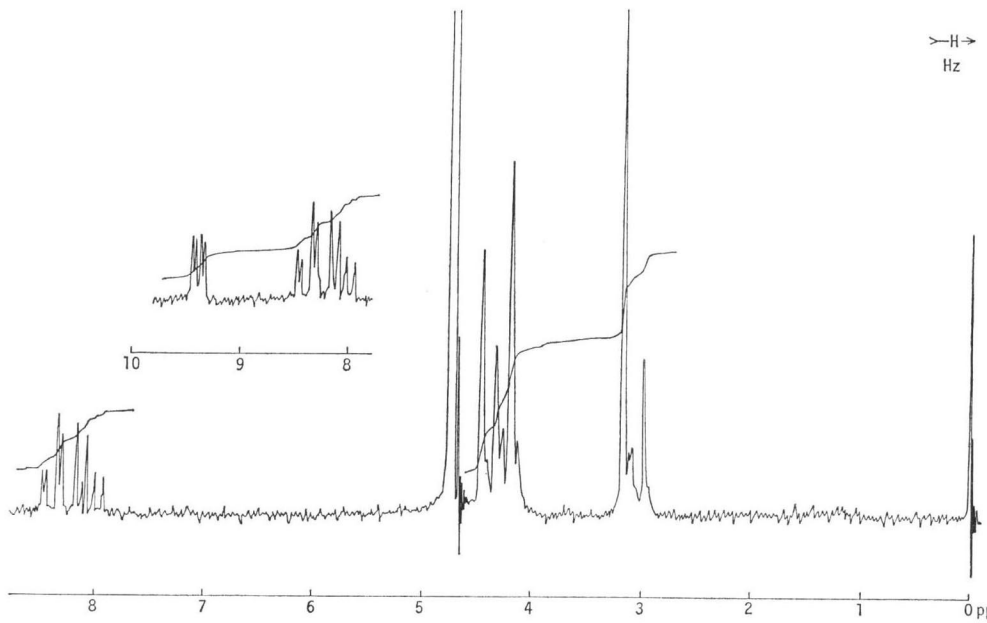
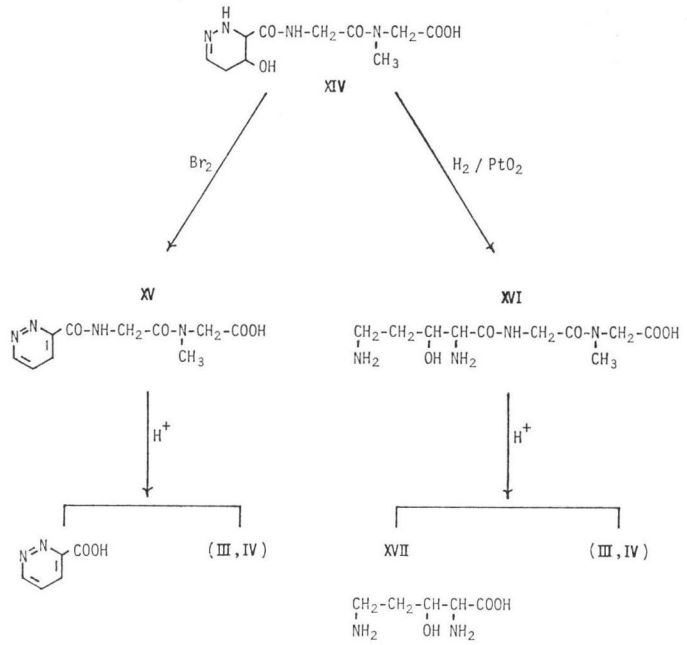
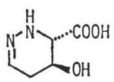


Chart 2. Structures of **XIV**, **XV**, **XVI** and **XVII**.



tions at 240 and 300 nm. The CMR of **XV** demonstrated the presence of four aromatic carbons and one carbonyl carbon in addition to the signals attributable to **III** and **IV**. As shown in the PMR of **XV** (Fig. 2), the splitting pattern of the aromatic protons indicated a 3-substituted-pyridazine structure. Upon acid hydrolysis, **XV** yielded **III**, **IV** and pyridazine-3-carboxylic acid[6]. The structure of **XV** is therefore 3-pyridazinecarbonylglycylsarcosine.

Catalytic hydrogenation of **XIV** over PtO_2 afforded a new basic peptide **XVI**. Acid hydrolysis of **XVI** in 6 N HCl liberated a new basic amino acid **XVII** ($C_5H_{12}N_2O_3$) along with **III** and **IV**. **XVII** was identified as L-*erythro*-β-hydroxyornithine from its optical rotation value ($[\alpha]_D$ +19.9°) and by direct comparison (TLC, PPC) with a racemic sample[7]. Thus, the structure of **X**, including its stereochemistry, was determined to be *trans*-(3*S*,4*S*)-4-hydroxy-2,3,4,5-tetrahydropyridazine-3-carboxylic acid.

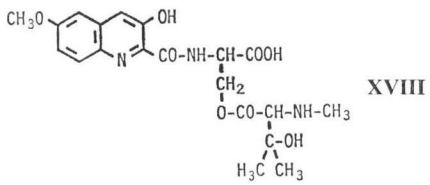


X

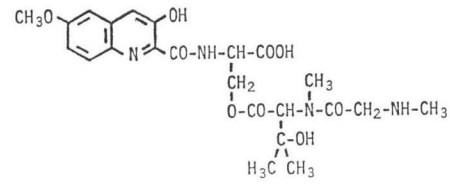
Reductive cleavage of hexahydropyridazine-3-carboxylic acid to ornithine has been described[8]. Structures of **XIV**, **XV**, **XVI** and **XVII** are shown in Chart 2.

Isolation of Depsipeptide Fragments

Under a specific hydrolysis condition (6 N HCl, 100°C, 3 hours), BBM-928 C afforded three solvent-extractable, UV-absorbing fragments, **XII**, **XVIII** ($C_{20}H_{25}N_3O_8$) and **XIX** ($C_{23}H_{30}N_4O_9$), all of which contained the same chromophore moiety, **V**. The IR spectra of **XVIII** and **XIX** indicated the presence of ester carbonyl group ($\nu_{C=O}$ 1,745 cm^{-1}). Upon mild alkaline hydrolysis, **XVIII** yielded **I** and **XII**, while **XIX** afforded **I**, **III** and **XII**. Thus, the structures shown below were assigned to **XVIII** and **XIX**.



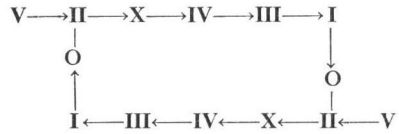
XVIII



XIX

Structure of BBM-928 A, B and C

It has been shown that BBM-928 C is composed of 2 moles of **VIII** cyclized in a symmetrical arrangement and that one mole each of six structural units (**I, II, III, IV, V** and **X**) constituted **VIII**. Isolation of peptide fragments, **XI, XII, XIII** and **XIV**, established the sequence of the six structural constituents in **VIII** as **V→II→X→IV→III→I**. Since the hydroxyl group of **II** was esterified by **I** in BBM-928 C as revealed by the isolation of depsipeptide fragments **XVIII** and **XIX**, the structure of BBM-928 C was established as that shown below in a schematic form:



BBM-928 A and B were shown to be diacetyl and monoacetyl derivatives of BBM-928 C, respectively. In a comparative PMR analysis, the OH-bearing methine proton of the tetrahydropyridazine moiety (**X**) of BBM-928 C was observed as a broad singlet at δ 4.25 ppm. This proton signal appeared at δ 5.52 ppm in BBM-928 A indicating that the acetylation took place on the hydroxyl group of the tetrahydropyridazine moiety. Thus the structures shown before are assigned to BBM-928 A, B and C.

Discussion

BBM-928 A, B and C are chromophoric cyclic depsipeptides containing 10 amino acid moieties and 2 moles of 3-hydroxy-6-methoxyquinaldic acid, a novel heterocyclic compound. BBM-928 is structurally related to the quinoxaline antibiotics which include echinomycin[9], quinomycins[10] and triostins[11]. The quinoxaline antibiotics are characterized by their cyclic depsipeptide structures which contain 8 amino acids and 2 moles of quinoxaline-2-carboxylic acid as a chromophore. Abundance of N-methyl amino acid moieties as structural constituents is also a common feature of BBM-928 and quinoxaline antibiotics. BBM-928 contains two unusual amino acids, L-β-hydroxy-N-methylvaline (**I**) and *trans*-(3*S*,4*S*)-4-hydroxy-2,3,4,5-tetrahydropyridazine-3-carboxylic acid (**X**), which have not been previously found in nature. In addition to the differences in amino acid and chromophore structures between BBM-928 and quinoxaline antibiotics, BBM-928 does not have a sulfur-containing intramolecular cross linkage which is present in the latter group.

As reported in our previous paper[2], BBM-928 A exhibited the highest antitumor activity among the three components. BBM-928 B, a monoacetyl analog, was about one-third as active as BBM-928 A, while BBM-928 C, a non-acetylated component, was almost devoid of antitumor activity. The PMR spectrum of BBM-928 indicated the presence of unequivalent methylene protons for glycine, sarcosine, serine and tetrahydropyridazine moieties. The difference in chemical shifts for the geminal proton signals was observed to a greater extent in BBM-928 A ($\Delta_{CH_\alpha\text{-}H_\beta}$ 0.48~2.07 ppm) than in BBM-928 C ($\Delta_{CH_\alpha\text{-}H_\beta}$ 0.13~1.70 ppm). This may reflect a conformational difference in the structures of BBM-928 A and C due to a participation of acetyl groups. The close relationship between biological activity and conformational state has been previously described for cyclic peptide antibiotics[12].

Experimental

Thin-layer chromatography (TLC) was performed on silica gel plate (Kieselgel $60F_{254}$, Merck) using the solvent systems shown below:

System No.	Solvent system	
S-123	MeOH - 10% $AcONH_4$ - 10% NH_4OH	(10: 9: 1)
N-114	*n*-BuOH-Acetone-AcOH-H_2O	(4: 5: 1: 1)
A-107	Phenol-H_2O	(4: 1)
SD-105	$CHCl_3$-*n*-PrOH-10% NH_4OH	(1: 4: 1)
SD-106	$CHCl_3$-MeOH-AcOH	(50: 50: 4)
SD-107	$CHCl_3$-MeOH	(9: 1)

PMR spectra were obtained on a JEOL C60HL or Varian FT80A spectrometer and CMR spectra by Varian FT80A apparatus operated in the FOURIER transform system. Ordinary mass spectra were measured on a Hitachi RMU-6MG mass spectrometer using the direct inlet probe. Amino acid analysis was carried out using a Hitachi 034-2U amino acid autoanalyzer.

Total acid hydrolysis of BBM-928

A solution of BBM-928 C (500 mg) in 20 ml of 6 N HCl was heated at 110°C for 20 hours in a sealed tube. The reaction mixture was diluted with 80 ml of water and extracted with *n*-BuOH (100 ml×2). The aqueous layer was concentrated under reduced pressure to give 415 mg of sticky solid. The solid was chromatographed on a column of Dowex 50W×4 (ϕ 1.5×120 cm) which was pre-equilibrated with 0.1 M pyridine-formic acid buffer (pH 3.1.) The column was developed with the same buffer solution (0.1 M, 480 ml) followed by a 0.2 M buffer (200 ml). The eluate was monitored by ninhydrin test and TLC (S-123). Appropriate fractions were combined and evaporated *in vacuo* to yield **I** (73 mg), **II** (37 mg) and **III** (45 mg) from eluates with 0.1 M buffer solution and **IV** (39 mg) from 0.2 M buffer eluate.

I: Colorless needles from aqueous EtOH, m.p. 260~261°C (dec.). $[\alpha]_D^{26}$ −4° (*c* 4.90, 5 N HCl). TLC: Rf 0.72 (S-123) and 0.40 (A-107). PMR $\delta_{DSS}^{D_2O}$ in ppm: 1.22 (3H, s), 1.43 (3H, s), 2.68 (3H, s) and 3.37 (1H, s). CMR $\delta_{DSS}^{D_2O}$ in ppm: 23.9 (q), 28.4 (q), 34.0 (q), 70.7 (s), 73.9 (d) and 171.6 (s). *Anal.* Calcd. for $C_6H_{13}NO_3$: C 48.97, H 8.90, N 9.51. Found: C 48.90, H 9.06, N 9.46. pKa′: 2.3 and 9.5 (titration equivalent: 152). CD (*c* 0.05, 0.5 N HCl): $[\theta]_{250}$ 0, $[\theta]_{208}$ +3,503 (peak) and $[\theta]_{204}$ +3,380. ORD (*c* 0.09, 0.5 N HCl): $[\phi]_{250}$ +610, $[\phi]_{226.5}$ +2,420 (peak) and $[\phi]_{220}$ +1,810.

II: Colorless needles from aqueous EtOH, m.p. 222~224°C (dec.). $[\alpha]_D^{22.5}$ −5.3° (*c* 0.8, 5 N HCl). TLC: Rf 0.46 (S-123) and 0.11 (A-107). Identified as D-serine by IR and TLC.

III: Colorless needles from aqueous EtOH, m.p. 208~210°C (dec.). TLC: Rf 0.45 (S-123) and 0.34 (A-107). MS: *m/z* 89 (M^+), 46, 44. *Anal.* Calcd. for $C_3H_7NO_2$: C 40.44, H 7.92, N 15.92. Found: C 39.90, H 8.21, N 15.68. Identified as sarcosine by IR and TLC.

IV: Colorless powder. TLC: Rf 0.49 (S-123) and 0.16 (A-107). Identified as glycine by IR and TLC.

The *n*-BuOH extracts described above were evaporated under reduced pressure to afford 150 mg of yellowish residue which was chromatographed on a column of silica gel (ϕ 2.1×50 cm). The column was developed with a mixture of $CHCl_3$ - *n*-PrOH - 10% NH_4OH, the elution being monitored by absorption at 345 nm. Appropriate fractions were concentrated *in vacuo* to give **V** (68 mg) from eluate with $CHCl_3$ - *n*-PrOH - 10% NH_4OH (6: 4: 0.2) and **VI** (35 mg) from eluate of the same solvent system with a ratio of 2: 8: 0.2.

V: Yellowish needles from MeOH, m.p. 223~225°C. TLC: Rf 0.43 (SD-105). λ_{max}^{MeOH} 225 nm (ε 33,100), 256 (30,200) and 350 (12,700). MS: *m/z* 219 (M^+), 201, 175, 173, 147, 132. PMR $\delta_{DSS}^{D_2O+NaOD}$ in ppm: 3.69 (3H, s), 6.89 (1H, d-d, *J*=10.5 & 1.9 Hz), 6.91 (1H, d, *J*=1.9), 7.05 (1H, s) and 7.60 (1H, d, *J*=10.5 Hz). Positive NOE (*ca.* 20%) observed on two protons at δ 6.89 and 6.91 upon irradiation at δ 3.69. *Anal.* Calcd. for $C_{11}H_9NO_4$: C 60.27, H 4.14, N 6.39. Found: C 60.20, H 4.03, N 6.21.

VI: Yellow needles, m.p. 255~257°C (dec.). TLC: Rf 0.37 (SD-105). λ_{max}^{MeOH} 226 nm (ε 36,900), 250 (25,400) and 340 (9,500). MS: *m/z* 205 (M^+), 187, 161, 159. PMR $\delta_{DSS}^{D_2O+NaOD}$ in ppm: 6.52 (1H,

d, J=2.0 Hz), 6.76 (1H, d-d, J=10.5 & 2.0 Hz), 6.90 (1H, s) and 7.52 (1H, d, J=10.5 Hz). *Anal.* Calcd. for $C_{10}H_7NO_4$: C 58.54, H 3.44, N 6.83. Found: C 57.69, H 3.83, N 5.76.

6-Methoxy-3-quinolinol (**VII**)

V (100 mg) was heated at 225~240°C for 1 minute on a metal bath. Brown residue was taken up in 20 ml of MeOH and insoluble material removed by filtration. Evaporation of the filtrate gave pale yellow solid which was chromatographed on a column of silica gel (ϕ 1.5×45 cm). The column was eluted with $CHCl_3$ - MeOH (99: 1) and eluation monitored by absorption at 365 nm. UV-absorbing fraction was evaporated *in vacuo* to afford **VII** (58 mg, 6-methoxy-3-quinolinol). Colorless plates from $CHCl_3$ - MeOH. m.p. 215~216°C. TLC: Rf 0.40 (SD-107). λ_{max}^{MeOH}: 224 nm (ε 44,800), 318 (6,800), 324 (6,800) and 333 (10,200). *Anal.* Calcd. for $C_{10}H_9NO_2$: C 68.56, H 5.18, N 8.00. Found: C 68.34, H 5.11, N 7.86.

Mild alkaline hydrolysis of BBM-928 C–Isolation of **VIII**

A solution of BBM-928 C (5.3 g) in 0.1 N NaOH (260 ml) was stirred at room temperature for 3 hours. The solution was acidified to pH 2.0 with 6 N HCl and extracted with two 200-ml portions of *n*-BuOH. The extracts were combined, washed with water (300 ml) and concentrated *in vacuo*. Yellowish residue was chromatographed on a column of silica gel (ϕ 2.5×40 cm). The column was developed with $CHCl_3$ - *n*-PrOH - 10% NH_4OH (2: 8: 0.4) and the elution monitored by TLC (N-114) and UV absorption at 345 nm. Fractions showing an Rf 0.27 spot were combined and evaporated *in vacuo* to afford **VIII** (4.09 g). λ_{max}^{MeOH} 232 nm (ε 36,400), 260 (26,500) and 345 (10,400). Drastic acid hydrolysis gave **I**, **II**, **III** and **IV**. DAKIN-WEST degradation and subsequent acid hydrolysis did not afford **I**.

A solution of **VIII** (570 mg) in THF (50 ml) was treated with a large excess of diazomethane solution. After evaporation of the solvent, the residue was chromatographed on a column of silica gel (ϕ 3.0×60 cm) to give **IX** (196 mg). TLC: Rf 0.68 (N-114). MW: 681 (osmometry in MeOH). MS: m/z 654 ($M^+ - H_2O - CH_3O$), 614, 512, 442, 408. *Anal.* Calcd. for $C_{31}H_{41}N_7O_{12} \cdot H_2O$: C 51.58, H 6.01, N 13.58. Found: C 52.10, H 5.93, N 12.38.

Mild acid hydrolysis of **VIII**—Isolation of **XI**

A solution of **VIII** (4.0 g) in 200 ml of 0.05 N HCl was heated under reflux for 2 hours and the resulting solution was extracted with two 200-ml portions of *n*-BuOH. Evaporation of the extract *in vacuo* gave yellowish solid which was chromatographed on a silica gel column (ϕ 3.0×50 cm). The column was developed with $CHCl_3$ - MeOH - AcOH (100: 6: 2) and the elution monitored by absorption at 345 nm and TLC (N-114). Appropriate fractions were evaporated *in vacuo* to afford yellowish powder of **XI** (2.75 g). TLC: Rf 0.42 (N-114). λ_{max}^{MeOH} 233 (ε 40,700), 260 (28,000) and 345 (11,500). Complete acid hydrolysis gave **II**, **III** and **IV**. **III** was determined to be the C-terminal of **XI** by DAKIN-WEST method.

Aqueous layer of the hydrolyzate was evaporated to give 1.2 g of sticky solid which contained **I**.

Mild alkaline hydrolysis of **VIII**—Isolation of **XII** and **XIII**

A solution of **VIII** (1.35 g) in 130 ml of 0.1 N NaOH was kept standing at 37°C for 2 days. The mixture was neutralized by Amberlite IRC-50 (H^+) and extracted with *n*-BuOH (200 ml×2). Evaporation of the extracts gave yellowish residue which was chromatographed on a Sephadex LH-20 column (ϕ 3.0×50 cm) using MeOH as eluant. UV-absorbing fractions were combined and concentrated to give **XII** (450 mg). The aqueous layer was concentrated and lyophilized to yield amorphous powder of **XIII** (700 mg, monosodium salt). Acid hydrolysis of **XIII** with 6 N HCl gave **I**, **III** and **IV**.

XII: Colorless needles from MeOH, m.p. 189~191°C. TLC: Rf 0.44 (N-114). λ_{max}^{MeOH} 227 nm (ε 34,300), 261 (25,400) and 345 (11,300). MS: m/z 306 (M^+), 288, 261, 202, 173. PMR $\delta_{TMS}^{DMSO\text{-}d_6}$: 3.86 (2H, m), 3.88 (3H, s), 4.53 (1H, m), 7.18 (1H, d, J=2.0 Hz), 7.21 (1H, d-d, J=10.5 & 2.0 Hz), 7.63 (1H, s), 7.84 (1H, d, J=10.5 Hz), 8.98 (NH, lost with D_2O shake). *Anal.* Calcd. for $C_{14}H_{14}N_2O_6$: C 54.90, H 4.61, N 9.15. Found: C 54.86, H 4.54, N 9.18.

XIII: TLC: Rf 0.10 (SD-106). PMR $\delta_{DSS}^{D_2O}$: 1.17 (3H, s), 1.30 (3H, s), 2.31 (2H, m), 3.03 (3H, s), 3.06 (3H, s), 3.6~4.5 (6H, m), 4.78 (1H, s), 6.73 (1H, t). *Anal.* Calcd. for $C_{16}H_{27}N_5O_7 \cdot Na \cdot H_2O$: C 43.53, H 6.39, N 15.86, Na 5.21. Found: C 43.14, H 6.76, N 15.62, Na 4.99.

Mild alkaline hydrolysis of **XI**—Isolation of **XII** and **XIV**

XI (2.57 g) was treated with 0.1 N NaOH at 37°C for 2 days. The hydrolyzate was neutralized and extracted with *n*-BuOH to afford 1.2 g of yellowish solid which contained **XII**. The aqueous layer was concentrated *in vacuo* to oily residue which was chromatographed on a column of Diaion HP-20 (ϕ 2.0×40 cm). The column was developed with water and the eluate monitored by ninhydrin test. Concentration of ninhydrin-positive fractions gave white amorphous powder of **XIV** (1.35 g). TLC: Rf 0.28 (SD-106). MS: *m/z* 255 (M^+-OH), 226, 183, 165, 128. PMR $\delta_{DSS}^{D_2O}$ in ppm: 2.30 (2H, m), 3.02 (3H, s), 3.55~4.4 (6H, m), 6.72 (1H, t). Complete acid hydrolysis of **XIV** gave **III** and **IV**.

Preparation of glycylsarcosine

To a stirred solution of N-carbobenzyloxy(Cbz)-glycine (1.04 g) and methyl sarcosinate (698 mg) in 45 ml of CH_2Cl_2, was added dropwise a solution of dicyclohexylcarbodiimide (DCC, 1.135 g) in 15 ml of CH_2Cl_2 at 0°C. The stirring was continued at 5°C for 16 hours and then 0.1 ml of AcOH was added to decompose excess DCC. The reaction mixture was filtered. The filtrate was washed with 0.1 N HCl followed by saturated $NaHCO_3$ solution and dried over anhydrous sodium sulfate. Evaporation of the solvent yielded methyl Cbz-glycylsarcosinate as oily solid (1.5 g). A part of the solid (492 mg) was kept standing at 0°C with a mixture of EtOH (3 ml) and 1 N NaOH (3.3 ml). The mixture was washed with EtOAc and the aqueous layer was acidified to pH 2.0 and extracted with EtOAc. The EtOAc extract was concentrated *in vacuo* to leave a white residue which was crystallized from benzene and ether. Cbz-glycylsarcosine, 305 mg, m.p. 106~107°C. *Anal.* Calcd. for $C_{13}H_{16}N_2O_5$: C 55.71, H 5.75, N 9.99. Found: C 56.01, H 5.68, N 9.90. A solution of Cbz-glycylsarcosine (194 mg) in 13 ml of EtOH was hydrogenated with 10% paradium on carbon (80 mg) under one atmospheric hydrogen pressure for 19 hours. The catalyst was removed by filtration and the solution evaporated to give 110 mg of glycylsarcosine. TLC: Rf 0.16 (N-114). MS: *m/z* 146 (M^+), 128, 117, 100, 89, 71, 57. PMR $\delta_{DSS}^{D_2O}$ in ppm: 2.97~3.03 (3H), 3.9~4.0 (4H, m).

Bromine oxidation of **XIV**—Preparation of **XV**

To a solution of **XIV** (298 mg) in 200 ml of $CHCl_3$ and 4 ml of AcOH, was added dropwise 200 mg of bromine at room temperature under vigorous stirring. Stirring was continued for one hour and the precipitate deposited was removed by filtration. The yellow filtrate was concentrated *in vacuo* with an addition of toluene to expedite AcOH removal. Lyophilization of the residue gave pale yellow powder of **XV** (160 mg). TLC: Rf 0.38 (SD-106). $\lambda_{max}^{H_2O}$ 245 nm (ε 1,610) and 305 (410). MS: *m/z* 253 (M^++1), 235, 165, 136, 107, 79. PMR $\delta_{DSS}^{D_2O}$ in ppm: 2.98~3.17 (3H), 4.0~4.5 (4H, m,), 8.04 (1H, d-d, J=8.5 & 5.0 Hz), 8.40 (1H, d-d, 8.5 & 1.7 Hz), 9.37 (1H, d-d, J=5.0 & 1.7 Hz).

Treatment of **XV** with excess diazomethane in ether afforded monomethyl ester of **XV** which was purified by silica gel chromatography. Colorless prisms, m.p. 173~173.5°C. MS: *m/z* 266 (M^+), 235, 164, 136, 107. λ_{max}^{MeOH} 245 nm (ε 2,100), 310 (160). *Anal.* Calcd. for $C_{11}H_{14}N_4O_4$: C 49.62, H 5.30, N 21.04. Found: C 49.82, H 5.04, N 20.93.

Acid hydrolysis of **XV**—Isolation of pyridazine-3-carboxylic acid

XV (10 mg) in 1 ml of 6 N HCl was heated at 105~110°C for 18 hours in a sealed tube. The solution was concentrated *in vacuo* to dryness which was triturated with 0.8 ml of water. Insoluble materials were collected by filtration, washed with water and dried *in vacuo* to give 3.1 mg of pyridazine-3-carboxylic acid, m.p. 195~197°C. $\lambda_{max}^{0.1N\ NaOH}$ 250 nm (ε 1,200) and 302 (300). $\lambda_{max}^{0.1N\ HCl}$ 245 (1,400) and 300 (200). IR spectrum identical with that of synthetic sample[6].

Catalytic reduction of **XIV**—Isolation of **XVI**

XIV (850 mg) was dissolved in a mixture of AcOH (2 ml), EtOH (3 ml) and water (7 ml), and hydrogenated over PtO_2 at room temperature for 72 hours. The catalyst was filtered off and the filtrate evaporated *in vacuo* to sticky residue. The solid was charged on a column of Amberlite CG-50 (NH_4^+, ϕ 3.0×50 cm) which was developed with water. The elution was followed by ninhydrin test and TLC (S-123). Appropriate fractions were collected and concentrated to afford 280 mg of **XVI**. TLC: Rf 0.41 (S-123). PMR $\delta_{DSS}^{D_2O+DCl}$ in ppm: 2.0 (2H, m), 3.06 (3H, s), 3.17 (2H, t), 3.55 (1H, d), 3.8~4.3 (5H, m).

Acid hydrolysis of **XVI**—Isolation of **XVII**

XVI (180 mg) in 10 ml of 6 N HCl was heated at 105°C for 16 hours in a sealed vessel. Evaporation of the reaction mixture yielded 210 mg of sticky solid which was chromatographed on a column of Amberlite CG-50 (70% NH_4^+, ϕ 1.2×25 cm). The column was first developed with 200 ml of water and then 0.3 N NH_4OH solution. The elution was monitored by ninhydrin test and TLC (S-123). After neutral fragments were eluted with water, **XVII** was eluted with 0.3 N NH_4OH. Appropriate fractions were concentrated *in vacuo* to yield white powder (96 mg) which was crystallized from aqueous HCl - EtOH to give colorless fine needles of **XVII** (71 mg). m. p. 255~256°C (dec.). $[\alpha]_D^{24}$ +19.9° (*c* 0.48, 2 N HCl). TLC: Rf 0.26 (S-123). PPC: Rf 0.26 (*n*-BuOH - MEK - c.NH_4OH - H_2O, 5: 3: 3: 1). MS: *m/z* 149 (M^+ +1), 130, 102, 86, 74. PMR $\delta_{DSS}^{D_2O}$ in ppm: 1.95 (2H, m), 3.17 (2H, t, *J*=7.3 Hz), 3.84 (1H, d, *J*=4.3 Hz), 4.22 (1H, m). *Anal.* Calcd. for $C_5H_{12}N_2O_3 \cdot HCl$: C 32.51, H 7.10, N 15.17, Cl 19.22. Found: C 32.59, H 7.11, N 14.93, Cl 18.41. Identified as *erythro*-β-hydroxyornithine by PMR and PPC comparison with authentic sample[7].

Isolation of **XII**, **XVIII** and **XIX**

A solution of BBM-928 C (500 mg) in 50 ml of 6 N HCl was refluxed for 3 hours. The reaction mixture was diluted with 50 ml of water and extracted with three 100-ml portions of *n*-BuOH. The extracts were combined, washed with water and evaporated *in vacuo*. The yellow residue was chromatographed on a column of Sephadex LH-20 (ϕ 3.0×50 cm) using MeOH as eluant. The eluate collected in 2-ml fractions was assayed by UV absorption (at 345 nm) and TLC (N-114). Fractions No. 60~74 which showed an Rf 0.40 spot by TLC were evaporated *in vacuo* to afford **XVIII** (46 mg). Concentration of fractions No. 108~155 gave **XIX** (23 mg) and fractions No. 207~220 yielded **XII** (35 mg). Complete acid hydrolysis of **XIX** gave amino acids **I**, **II** and **III**.

XVIII: Colorless needles from MeOH, m.p. 242°C. TLC: Rf 0.40 (N-114). λ_{max}^{MeOH} 229 nm (ε 42,000), 261 (28,300) and 345 (12,600). IR: ν_{max}^{KBr} 3370, 1745, 1660, 1627, 1235. MS: *m/z* 377 (M^+ −58), 302, 288, 202, 173. PMR $\delta_{TMS}^{DMSO\text{-}d_6}$ in ppm: 1.07 (3H, s), 1.15 (3H, s), 2.32 (3H, s), 3.27 (1H, s), 3.87 (3H, s), 4.4~4.8 (3H, m), 7.17 (1H, d), 7.19 (1H, d–d), 7.60 (1H, s), 7.78 (1H, d), 9.24 (NH). *Anal.* Calcd. for $C_{20}H_{25}N_3O_8 \cdot H_2O$: C 52.97, H 6.00, N 9.26. Found: C 53.32, H 5.90, N 9.27.

XIX: Amorphous pale yellow powder. TLC: Rf 0.10 (N-114). λ_{max}^{MeOH} 224 nm (ε 35,800), 262 (26,900) and 345 (10,700). IR: $\nu_{C=O}^{KBr}$ 1740 & 1660.

Acknowledgement

The authors wish to thank Prof. T. SHIBA of Osaka University for the sample of *erythro* and *threo*-β-hydroxyornithine and related information, and to Prof. N. IZUMIYA of Kyushu University for information about β-hydroxy-N-methylvaline. The authors are indebted to Prof. T. GOTO of Nagoya University for NOE data, and to Prof. M. OHASHI of the University of Electro-communication for mass spectroscopic analysis and valuable discussions. Thanks are also due to Drs. T. W. DOYLE and J. A. MATSON of Bristol Laboratories, Syracuse, New York, for field desorption mass spectrum and 360 MHz PMR data.

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